

THE ALLOPHANATES OF CERTAIN STEROLS

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THE ALLOPHANATES OF CERTAIN STEROLS.*

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Since Takahashi (1) reported the results of a study which led him to the conclusion that vitamin A (called by him biostearin) has the properties of a sterol, this class of substances has assumed unusual interest. More recently Hess (2) and Steenbock (3) and their associates have brought to light the fact that the substance in natural foods which can be activated by irradiation with ultra-violet light, so as to confer upon it the properties of vitamin D as manifested in the prevention or healing of the rachitic lesion, is contained in the sterol fraction. Hess and Windaus (4) have brought forward evidence that ergosterol is extraordinarily potent in its antirachitic effect after irradiation. Hess and Anderson (5) have found that α -sitosterol prepared from maize oil can also be so activated. The existence of numerous sterols in biological materials, some of which can be activated by light, makes it desirable to have effective methods for their separation and purification. The study of the allophanic esters of sterols was begun by us in 1925. The study of the preparation and properties of the esters described in this paper was undertaken with a view to discovering the possible usefulness of this class of derivatives in the isolation of fat-soluble vitamins. It seemed desirable to determine whether sterols would react as do other alcohols with cyanic acid with the formation of allophanic esters. The properties of allophanates of other alcohols have in general been favorable for identification (6). If this should be true of the sterols we should have the advantage

* The experimental work reported in this paper was carried out by Miss Ume Tange and was submitted to the faculty of the Johns Hopkins University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

of working with easily formed derivatives containing nitrogen and possibly with solubility properties which would be advantageous in the separation and purification of this class of substances.

Allophanates of Sterols.

Preparation of Cyanuric Acid.—Cyanuric acid was prepared according to the procedure described by Béhal (7). 250 gm. of urea were heated to 110–131° while a rapid stream of chlorine gas was passed into it. The chlorine was generated by treating potassium permanganate with hydrochloric acid. The gas was first washed with water, then with sulfuric acid. The excess of chlorine not absorbed by the urea was absorbed by sodium hydroxide. On passing chlorine into urea the temperature rises rapidly, and care must be taken that it does not rise above 131° during the first period of the reaction, since urea is sublimed without being attacked by the chlorine. At 131° the urea melts with a rapid evolution of gas, after which the resulting material gradually solidifies. As the solidification progresses the temperature is gradually raised to 150°, and the material is treated with chlorine gas until the contents of the flask become solid. This requires 4 to 5 hours. The white solid product is then heated at 190–240° for 12 to 20 hours, or until no further evolution of gas takes place. The length of the heating period at these high temperatures varies greatly and depends upon the temperature maintained while the chlorine gas was being introduced.

The crude cyanuric acid was treated with 2 liters of boiling water and 200 ml. of concentrated ammonium hydroxide. This operation was repeated until the solution no longer yielded copper cyanurate on the addition of cupric ammonio sulfate, $\text{Cu}(\text{NH}_3)_4\text{SO}_4$. The cupric cyanate was suspended in water and treated with dilute nitric acid which decomposed it into cupric nitrate and cyanuric acid. The yield in several preparations was 50 to 60 per cent of the theoretical. If the heating is too long continued at the higher temperatures employed toward the end of the operation, amorphous insoluble "caluret," $\text{C}_4\text{H}_8\text{O}_7\text{N}_6$, is produced and the yield is reduced.

The cupric ammonio sulfate solution employed was prepared by dissolving $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water, and adding ammonium

hydroxide until the precipitate first formed nearly but not quite redissolves.

Cholesteryl Allophanate.—3 gm. of cholesterol melting at 148°, prepared from cod liver oil, were dissolved in 120 ml. of benzene. Into the solution, kept in a freezing mixture, cyanic acid gas was passed by means of a current of dry carbon dioxide. The latter was first washed with water, then with sulfuric acid, and lastly passed through a calcium chloride tube. The cyanic acid gas was prepared by heating cyanuric acid to dissociation. Since cyanic acid has but a transitory existence, the tube in which it is generated and the tube carrying the gas into the benzene solution of the

TABLE I.
Solubility of Cholesteryl Allophanate in Several Solvents.

Solvent.	Temperature of solution.	Time.	Amount dissolved in 100 ml.
	°C.	min.	gm.
Ether.....	35-38	10	0.009
Petroleum ether.....	40-45	10	Practically insoluble.
CHCl ₃	60-65	10	0.492
CCl ₄	77 (about).	10	0.049
C ₂ H ₄ Cl ₂	80-81	10	0.230
C ₆ H ₆	80-82	10	0.142
C ₆ H ₅ CH ₃	90-93	10	0.110 (?)*
Ethyl alcohol.....	80-82	10	0.053
Acetone.....	56-58	10	0.023
Pyridine.....	96 (about).	10	0.780

* During filtering, it gelatinized on filter paper.

sterol must be kept suitably heated to prevent polymerization to cyanuric acid. The proportion of cyanuric acid dissociated to the sterol employed should be 3:1 to 5:1.

The reaction product of cholesterol and cyanic acid forms a pasty mass. It was allowed to stand overnight in contact with the cyanic acid dissolved in the benzene, then filtered with suction. The material was washed with benzene, then with petroleum ether, in both of which it is nearly insoluble. The crude product was recrystallized from pyridine. The yield was about 65 per cent of the theoretical.

From chloroform or pyridine cholesteryl allophanate crystal-

lizes as colorless, shining needles which melt at 235–236°. It is insoluble in water and but slightly soluble in other common solvents (see Table I). The nitrogen content (Kjeldahl) of the crystals was 28.63 per cent (average of three determinations). The calculated nitrogen content of the reaction product of cholesterol with 1 molecule of allophanic acid ($C_{29}H_{48}N_2O_3$) is 28.02 per cent. The product obtained was, therefore, cholesteryl allophanate, $C_{27}H_{45}O-CO-NH-CO-NH_2$.

No detailed study has been made of the time required to saponify cholesteryl allophanate completely by means of alkaline reagents. The ester is hydrolyzed slowly by 10 per cent alcoholic KOH, and more rapidly by sodium ethylate.

0.24 gm. of cholesteryl allophanate was treated in an Erlenmeyer flask with 35 ml. of 10 per cent KOH (95 per cent alcohol), and heated in a water bath under a condenser for 1 hour. Ammonia gas was evolved. After cooling the flask, water was added which caused the separation of cholesterol. The latter was shaken out with 3 portions of ether. The combined ethereal extracts were washed with water, and the ether removed by distillation. The residue was pale yellow in color. Recrystallization from 95 per cent alcohol yielded the typical crystalline form of cholesterol, and the product melted at 148°. 56 per cent of the cholesterol was recovered. That some of the allophanic acid still remained as potassium allophanate in the water from which the cholesterol was extracted was shown by the evolution of carbon dioxide on acidifying it with HCl. The salts of allophanic acid are fairly stable but the free acid at once decomposes into carbon dioxide and urea. From these observations there can be no doubt that the product described is the allophanic ester of cholesterol.

Cholesteryl allophanate is very little hydrolyzed by alcoholic hydrochloric acid.

0.222 gm. of cholesteryl allophanate was treated with 35 ml. of 2.5 per cent sodium ethylate solution according to the method of Kossel and Obermüller (8). The solution was then diluted with water and shaken out with 3 portions of ether. The washed ether was evaporated. 80 per cent of the cholesterol was recovered by this method. When a 5 per cent solution of sodium ethylate was employed the yield of cholesterol was increased to 85 per cent.

There forms on saponification with sodium ethylate a sticky material which makes the resulting cholesterol less easily purified than when alcoholic KOH is used. Sodium methylate proved less satisfactory than sodium ethylate for the saponification of cholesteryl allophanate.

Carbon tetrachloride proved as satisfactory as benzene as a solvent in which to form cholesteryl allophanate by the action of cyanic acid upon the sterol.

Sitosteryl Allophanate.—Sitosterol, having a melting point of 137° , was prepared by the ordinary method from soy bean oil. The yield was 0.1 to 0.12 per cent. Its color reactions and solubility agreed with Burian's description (9). 3 gm. of sitosterol were

TABLE II.
Solubility of Sitosteryl Allophanate.

Solvent.	Temperature of digestion mixture.	Time.	Amount dissolved in 100 ml.
	$^{\circ}\text{C}.$	<i>min.</i>	<i>gm.</i>
Ether.....	35-38	10	0.020
Petroleum ether.....	40-45	10	Trace.
CHCl_3	60-65	10	0.125
$\text{C}_2\text{H}_4\text{Cl}_2$	80-81	10	0.107
C_6H_6	80-82	10	0.065
$\text{C}_6\text{H}_5\text{CH}_3$	90-93	10	0.068
Ethyl alcohol.....	80-82	10	0.061
Acetone.....	56-58	10	0.078
Amyl alcohol.....	93-95	10	0.220

treated with cyanic acid gas in benzene according to the procedure described for preparing cholesteryl allophanate. The separation of the reaction product was accomplished in an analogous manner. Sitosteryl allophanate is very insoluble in any of the ordinary solvents (Table II). Amyl alcohol proved the best solvent from which to recrystallize it. The nitrogen content by the Kjeldahl method was 30.09 and 30.10 per cent in two determinations (theoretical 28.02 per cent), but the manner of preparation and the behavior of the substance on hydrolysis leave no room for doubt that sitosteryl allophanate, somewhat contaminated, was formed.

On saponification for 1 hour with 10 per cent alcoholic KOH,

60 per cent of the sitosterol was recovered in a pure condition (m.p. 136–137°). It is very stable toward mineral acids. From amyl alcohol it separates as colorless crystals which appear under the microscope as pinwheel groups of needles radiating from a common center. It melts at 246–247° with slight charring.

Dihydrocholesteryl Allophanate.—Dihydrocholesterol was prepared by the reduction of cholesterol in ethereal solution by hydrogen in the presence of platinum black. We proceeded according to the method of Willstätter and Mayer (10). The product melted at 141.5–142°. We had no difficulty in causing 1 gm. of cholesterol with 0.1 gm. of platinum black to absorb the theoretical amount of hydrogen in 4 to 5 hours, whereas Burian and Mayer, using one-third as much platinum black as cholesterol, required 2 days for saturation of the sample.¹ The generation of hydrogen by means of zinc and sulfuric acid can be greatly accelerated by placing the zinc in a solution of copper sulfate before using. The hydrogen was washed in 2 per cent KMnO_4 and concentrated pyrogallie acid in 50 per cent KOH before bringing it into contact with the sterol.

Dihydrocholesteryl Allophanate.—This derivative was formed in the manner already described for cholesterol. It separates from benzene in a pasty mass, on standing overnight. In solubility it resembles cholesteryl allophanate (Table III). The yield was about 65 per cent of the theoretical. It is readily recrystallized from pyridine in hair-like forms. It melts at 255–256°. Like the other allophanates of sterols the nitrogen determinations tended to run high. Two samples were found to contain 29.76 and 29.65 per cent of N (calculated 28.02 per cent), indicating that it was not entirely pure.

Dihydrocholesteryl allophanate is more readily hydrolyzed by 10 per cent alcoholic KOH than is cholesteryl allophanate. After

¹ In a study of the quality of different samples of platinum blacks, Dr. Tange (unpublished data) has found that the quality can be determined by its power to reduce an ethereal solution of *p*-benzoquinone. At least three grades can be distinguished. Some can reduce benzoquinone to hexahydroquinone, some to hydroquinone, and some preparations produce intermediate stages of reduction. She attributed the special properties of her samples of platinum black to the temperature employed in reducing platonic chloride with formaldehyde.

1 hour's hydrolysis practically 100 per cent of the calculated amount of dihydrocholesterol was recovered. The sample thus recovered melted at 141° .

Coprosterol.—Coprosterol was prepared from the feces of sixteen rats which were fed during 3 weeks exclusively on raw hog brains. They consumed about 45 pounds of fresh brain. Each day the feces were collected and placed in 95 per cent alcohol. The ether-soluble matter from the feces was obtained by shaking repeatedly with ether in a wide mouthed bottle, and decanting off the supernatant ether so long as anything was extracted. 400 gm. of a brownish, semisolid material were thus secured. This was saponified in a water bath for 45 minutes by means of 20 per

TABLE III.
Solubility of Dihydrocholesteryl Allophanate.

Solvent.	Temperature of digestion mixture.	Time.	Amount dissolved in 100 ml.
	$^{\circ}\text{C}.$	<i>min.</i>	<i>gm.</i>
Ether.....	35-38	10	0.010
Petroleum ether.....	40-45	10	Practically insoluble.
CHCl_3	60-65	10	0.0135
$\text{C}_2\text{H}_4\text{Cl}_2$	80-81	10	0.045
C_6H_6	80-82	10	0.025
$\text{C}_6\text{H}_5\text{CH}_3$	90-93	10	0.040
Ethyl alcohol.....	80-82	10	0.020
Acetone.....	56-58	10	0.007
Pyridine.....	96 (about).	10	0.720

cent KOH in 95 per cent alcohol, 200 ml. of the solution to each 100 gm. of crude lipoid material being used. The alcohol was distilled off and water was added to the residue and shaken. Ether was then added and shaken to remove the lipoids. 4 portions of ether were used. The ethereal extract was washed with water and treated with animal charcoal. This removed most of the fecal odor and much of the color. From the ethereal solution 30 to 35 gm. per 100 gm. of feces, of a pale brownish syrup, were secured. On crystallizing from 95 per cent alcohol, several fractions were separated. On recrystallization colorless crystals were secured from each fraction, which consisted of large needles. No typical cholesterol crystals could be seen. Eventually, on recrystallizing

from 90 per cent alcohol, fractions were secured which melted at 95–96°, 100°, 103–104°, and 110°. The largest fraction melted at 103–104°. The yield was 7.5 gm. It gave the yellow to orange-red color of Salkowski's reaction.

Coprosteryl Allophanate.—This was prepared from coprosterol, m.p. 103–104°, in the same manner as were other sterol allophanates. The reaction appears to progress slower than that of the other sterols studied, so it was left in the benzene in contact with cyanic acid for 2 or 3 days. The odor of cyanic acid still persisted at that time. Coprosteryl allophanate is moderately soluble in cold benzene, so it would probably be best to select another

TABLE IV.
Solubility of Coprosteryl Allophanate.

Solvent.	Temperature of digestion mixture.	Time.	Amount dissolved in 100 ml.
	°C.	min.	gm.
Ether.....	35–38	10	0.170
Petroleum ether.....	40–45	10	0.006
CHCl ₃	Room.	10, shaken.	2.000 (about).
C ₂ H ₄ Cl ₂	80–81	10	2.540
“.....	Room.	10, shaken.	0.320
C ₆ H ₆	80–82	10	2.610
“.....	Room.	10, shaken.	0.300
C ₆ H ₅ CH ₃	90–93	10	2.790
“.....	Room.	10, shaken.	0.225
Ethyl alcohol.....	80–82	10	0.213
Acetone.....	56–58	10	0.223

solvent. It was filtered off and washed with petroleum ether. Finally it was dissolved in a small amount of benzene and filtered hot. To the filtrate was added an equal volume of petroleum ether. On standing, needle crystals with a silvery luster separated, which melted at 210–211°. The yield was about 60 per cent of the theoretical. The nitrogen content as determined by Kjeldahl analysis was 28.8 and 28.97 per cent (theoretical value 28.02 per cent).

Hydrolysis of Coprosteryl Allophanate.—With 10 per cent alcoholic KOH, after $\frac{1}{2}$ hour's digestion, 80 per cent of the theoretical amount of coprosterol in the sample of allophanate was re-

covered in the characteristic needles melting at 104° . The substance is more easily hydrolyzed by 10 per cent alcoholic HCl, than any of the other allophanates studied. It was estimated that 10 per cent was hydrolyzed by this reagent in $\frac{1}{2}$ hour. Coprosteryl allophanate is easily soluble in chloroform in the cold and somewhat less soluble in carbon tetrachloride (Table IV). It separated in a gelatinous mass from concentrated solutions in carbon tetrachloride. In other cold organic solvents it is not readily soluble. Its solubility in hot chloroform is greater than that of any other allophanate of a sterol which we have prepared.

Isocholesterol.—Isocholesterol was prepared from wool fat by saponification with sodium ethylate since these lipoids are not attacked by alcoholic KOH except when heated under pressure. The procedure was the usual one and will not be described. The final product was identified as ischolesterol by its crystalline form, its melting point, $137-138^{\circ}$, and its color reactions.

Isocholesteryl Allophanate.—Isocholesteryl allophanate was prepared in the usual manner. After standing for several days at room temperature in benzene containing cyanic acid, the white mass which separated was mixed with petroleum ether and filtered, washed with petroleum ether, then digested several times in benzene. The product, freed from benzene, was dissolved in cold pyridine and filtered. Water was added until no more precipitate separated. This process was repeated three times more. The white product was washed thoroughly with hot water to remove pyridine. After drying it was boiled with petroleum ether to remove any unesterified ischolesterol. No satisfactory solvent was found from which to crystallize this allophanate. Nitrogen determinations gave 28.21 and 28.12 per cent as compared with 28.02 per cent, the theoretical value. Hydrolysis with 10 per cent alcoholic KOH for 1 hour resulted in the recovery of 70 per cent of the theoretical amount of ischolesterol. The solubility of ischolesteryl allophanate differs remarkably from any others we have prepared. It is easily soluble in ether, carbon disulfide, chloroform, carbon tetrachloride, ethylene dichloride, amyl alcohol, and pyridine in the cold. It is completely precipitated from its pyridine solution by the addition of water. It is fairly soluble in benzene and toluene in the cold, and very readily in the hot solvents. From hot benzene solutions it gelatinizes on cooling.

TABLE V.
Color Reactions of Sterol Allophanates.

Test No.	Reagent.	Substance.				
		Cholesteryl allophanate.	Dihydrocholesteryl allophanate.	Sitosteryl allophanate.	Coprosteryl allophanate.	
1	Concentrated H_2SO_4 .	Yellowish red with green fluorescence.	Pale yellow with faint green fluorescence.	Similar to cholesteryl allophanate.	Pale purple-red → brownish red with green fluorescence.	Reddish yellow → yellowish red with some green fluorescence.
2	Salkowski.	Yellow → yellowish red → cherry-red → purple-red with green fluorescence.	Fine ring of pale yellow on junction of two layers.	“ “	Yellow → orange-red with faint green fluorescence.	Fine ring of reddish yellow on junction of two layers.
3	Liebermann-Burchard.	Violet → blue → bluish green → green.	No reaction.	“ “	Pale blue → bluish green → green.	Yellow → reddish yellow, strongly marked green fluorescence.
4	Schiff.	Yellow → yellowish red.	“ “	“ “	Yellow → reddish brown.	Yellow → beautiful greenish yellow → reddish brown.
5	HCl and $FeCl_3$.	Violet → deep blue.	Violet → deep blue.	“ “	Violet → deep blue.	Violet → deep blue.
6	$SbCl_5$.	Cobalt-blue.	Cobalt-blue.	“ “	Cobalt-blue.	Negative.*

* The black mass produced by $SbCl_5$ was insoluble in $CHCl_3$.

It is more sparingly soluble in ethyl and methyl alcohol, and in nitromethane, and is insoluble in petroleum ether.

Among the cholesterol color tests, the following were applied: (1) To a small quantity of the substance about 0.5 cc. of concentrated H_2SO_4 was added. (2) A small amount of a sample was dissolved in 1 cc. of CHCl_3 ; then an equal volume of concentrated H_2SO_4 was added (Salkowski reaction). (3) A minute quantity of a sample was dissolved in 1 cc. of CHCl_3 ; then an equal amount of acetic anhydride and 2 or 3 drops of concentrated H_2SO_4 were added (Liebermann-Burchard reaction). (4) A small amount of a sample was warmed in a porcelain dish with a few drops of concentrated HNO_3 ; then the dry substance was moistened with concentrated NH_4OH (Schiff reaction). (5) A small amount of a sample was triturated in a porcelain dish with a few drops of a mixture consisting of 3 volumes of concentrated HCl and 1 volume of a 10 per cent solution of ferric chloride. (6) Into a small amount of a sample, 1 or 2 drops of SbCl_5 were added. After standing for about 5 minutes, the liquid portion was decanted; then the dark sticky mass was washed with CCl_4 twice. The black mass so obtained was dissolved in CHCl_3 , yielding the characteristic cobalt-blue color.

The results of the color reactions developed in the above tests are given in Table V.

SUMMARY.

The allophanic acid esters of cholesterol, sitosterol, ischolesterol, dihydrocholesterol, and coprosterol have been produced through the action of cyanic acid upon the sterol dissolved in benzene.

The property of dissolving in fat solvents is in great measure lost when the sterol is converted into the allophanate.

The solubility of these sterols with the exception of ischolesterol in most solvents is so low that recrystallization can be effected with very little substance remaining in the mother liquor.

It appears probable that the allophanic esters of the sterols may prove advantageous in the separation of mixtures of sterols, and perhaps in the isolation of fat-soluble vitamins.

The solubilities in a number of ordinary solvents and the ease of saponification with alcoholic potassium hydroxide and with sodium ethylate have likewise been studied.

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